



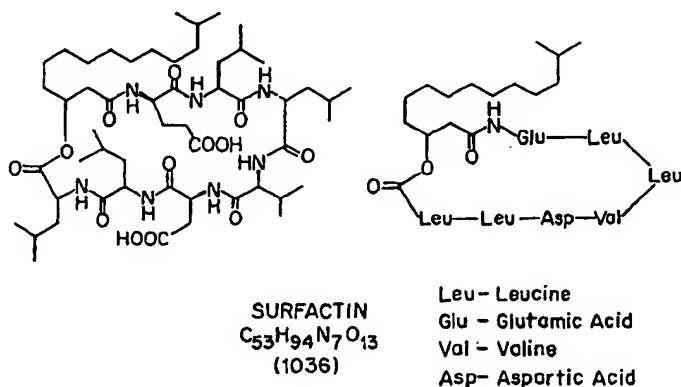
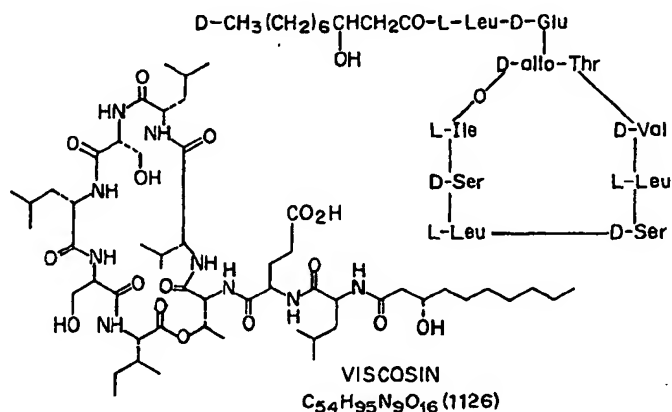
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12Q 1/48, 1/02	A1	(11) International Publication Number: WO 99/20792 (43) International Publication Date: 29 April 1999 (29.04.99)
(21) International Application Number: PCT/CA98/00970 (22) International Filing Date: 16 October 1998 (16.10.98) (30) Priority Data: 60/062,515 17 October 1997 (17.10.97) US (71) Applicant: TERRAGEN DIVERSITY INC. [CA/CA]; University of British Columbia, Suite 300, 2386 East Mall, Vancouver, British Columbia V6T 1Z3 (CA). (72) Inventors: DAVIES, Julian, E.; 4428 W. 6th Avenue, Vancouver, British Columbia V6R 1V3 (CA). WATERS, Barbara; 5706 Timbervale Road, Delta, British Columbia V4L 2E6 (CA). SAXENA, Geeta; #302-2875 Osoyoos Crescent, Vancouver, British Columbia V6T 2G3 (CA). (74) Agent: DEETH WILLIAMS WALL; National Bank Building, Suite 400, 150 York Street, Toronto, Ontario M5H 3S5 (CA).		(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published With international search report.

(54) Title: METHOD FOR INHIBITING EUKARYOTIC PROTEIN KINASES

(57) Abstract

A number of bacterial cultures were screened for the ability to inhibit sporulation using a strain *Streptomyces* 85E. Evaluations of the cultures which tested positive in this assay led to the discovery that lipopeptide biosurfactants such as surfactin and viscosin are effective inhibitors of eukaryotic protein kinase activity. Thus, a method for inhibiting eukaryotic protein kinase activity present in a sample or organism includes the step of adding to the sample or organism an effective inhibitory amount of a lipopeptide biosurfactant.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

- 1 -

METHOD FOR INHIBITING EUKARYOTIC PROTEIN KINASES

DESCRIPTIONBACKGROUND OF THE INVENTION

This application relates to a method for inhibiting eukaryotic protein kinases
5 using lipopeptide biosurfactant compounds such as surfactin or viscosin.

Kinase and phosphatase enzymes play important roles in the regulation of both
eukaryotic and prokaryotic cells. For example, in eukaryotic cells, the control of
proliferation and differentiation is achieved by multiple signal transduction pathways that
are regulated by the coordinated action of protein kinases and phosphatases.

10 Kinase activity in eukaryotes can be classified as one of three types: those
enzymes which phosphorylate tyrosine residues; those which are specific for serine or
threonine residues; and those which have dual specificity for both tyrosine and
serine/threonine residues. Because of the importance of these enzymes in eukaryotic
regulatory processes, it would be highly desirable to be able to inhibit kinases of the
15 various classes selectively to assist in the elucidation of kinase and phosphatase mediated
pathways, particularly those that may be of medical significance. In addition, selective
kinase or phosphatase inhibitors have potential uses as therapeutics. For example, it has
been reported that a tyrosine kinase blocker designated AG-940 specifically inhibits the
Jak-2 protein tyrosine kinase which is deregulated and constitutively activated in the
20 leukemic cells of acute lymphoblastic leukemia (ALL) patients. Meydan, et al. "Inhibition
of acute lymphoblastic leukaemia by a Jak-2 inhibitor", *Nature* 379: 645-648 (1996). This
inhibition induced changes in cells consistent with entry into apoptosis when tested *in*
vitro. Further, the intravenous administration of the inhibitor into mice previously injected
with ALL cells has been shown to be effective to eradicate the ALL cells from the marrow.

25 Notwithstanding the potential uses of kinase and phosphatase inhibitors, the
number of known and characterized inhibitors is quite small. Staurosporine and K-252a
are known to act as generalized kinase inhibitors, while herbimycin and radicicol
specifically inhibit tyrosine kinases, albeit with fairly low effectiveness. There are few if
any known specific inhibitors for the MAP kinase family, an important group of enzymes

- 2 -

thought to be central in the transmission of a wide variety of signals received at the cellular membrane to the transcriptional and replication machinery of the nucleus.

To facilitate the identification of new kinase and phosphatase inhibitors, we have developed an assay which is described in our prior US Patent No. 5,770,392 issued June 23, 1998, and in PCT Publication No. WO98/17822 claiming priority therefrom, which are incorporated herein by reference. This assay basically involves the steps of :

adding the material to be tested for kinase inhibitory activity to a growing culture of a prokaryotic organism such as a streptomycete;

allowing the culture to grow for a period of time in the presence of the material; and

observing the culture for altered development relative to development of the prokaryotic organism grown in the absence of the material. Observation of altered development is indicative that the material has activity as an inhibitor of post-translational protein phosphorylation. In particular, the material to be tested can be added to a growing culture of the prokaryotic organism by placing a carrier disk bearing the material on a freshly seeded plate. Inhibition of the development of aerial mycelia and spore formation is an indicator that the material has activity as an inhibitor of post-translational protein phosphorylation.

SUMMARY OF THE INVENTION

Using the assay method of our prior invention, we have screened a number of bacterial cultures for the ability to inhibit sporulation of our preferred tester strain *Streptomyces* 85E. Evaluations of the cultures which tested positive in this assay have led to the discovery that lipopeptide biosurfactants such as surfactin and viscosin are effective inhibitors of eukaryotic protein kinase activity. Thus, the present invention provides a method for inhibiting eukaryotic protein kinase activity present in a sample or organism comprising the step of adding to the sample or organism an effective inhibitory amount of a lipopeptide biosurfactant.

BRIEF DESCRIPTION OF THE FIGURES

Figs. 1A and 1B show the structures of viscosin and surfactin.

DETAILED DESCRIPTION OF THE INVENTION

5 Although eukaryotic and prokaryotic protein kinases generally have different substrate specificities, it has been observed that in some prokaryotic organisms eukaryote-like kinase and phosphatase activities may complement the two component systems typical of bacteria. In particular, streptomycetes, (Waters et al., "Protein tyrosine phosphorylation in streptomycetes", *FEMS Microbiology Letter* 120: 187-190 (1994); Li et al, "Cloning
10 purification and properties of a phosphotyrosine protein phosphatase from *Streptomyces coelicolor* A3(2)", *J. Bact.* 178: 136-142 (1996)); *Myxococcus xanthus* (Zhang et al., "Identification of a putative eukaryotic-like protein kinase family in the developmental bacterium *Myxococcus xanthus*", *J. Bact.* 174: 5450-5453 9192); and cyanobacterial species. (Zhang, C-C, "Bacterial signaling involving eukaryotic-type protein kinases",
15 *Molec. Microbiol.* 20: 9-15 (1996)). Because of this, certain microbes can be used to provide an assay for screening materials for activity as inhibitors of eukaryotic post-translational protein phosphorylation, and in particular for protein kinase activity.

 We have used a wild strain of *Streptomyces* isolated from soil and designated *Streptomyces* WEC478-85E (hereinafter strain 85E) to test culture supernatants from
20 various materials. Strain 85E has been deposited with the American Type Culture Collection in accordance with the provisions of the Budapest treaty and has been assigned Accession Number ATCC 55824.

 In the test assay, material to be tested was applied to a filter paper disk and then placed on a plate which has been freshly seeded with the strain 85E test organism.
25 The test organism is then allowed to grow in the presence of the filter paper disk for a period of 24 to 36 hours, after which the organism is evaluated for altered development in the zone around the disk. An observation of an inhibition of the formation of aerial mycelia and spores, without inhibition of the growth of vegetative mycelia is particularly indicative of the presence of an inhibitor of post-translational phosphorylation.

30 Once microbial cultures are identified which produce inhibitors of the type detected using the assay of the invention, the specific inhibitor that is produced by the

- 4 -

culture were isolated and characterized. In particular, two bacterial cultures, WEC64-11C and WEC297-60A, which have been identified as strains of *Pseudomonas* and *Bacillus*, respectively, produce cell free supernatants which inhibit sporulation and formulation of aerial mycelia in tester strain 85E and other strains of *Streptomyces*. For each culture, an ethyl acetate extract was prepared by separately extracting the supernatant and the cells and combining the resulting products and this extract was fractionated on a silica gel column. Activity in the fractions recovered from the column was assayed to permit identification of an active inhibitory fraction. This fraction was further purified by chromatography, and the inhibitory compound was analyzed by mass spectroscopy.

In the case of the *Pseudomonas* 11C, the product was determined to be viscosin. In the case of *Bacillus* 60A, the product was determined to be surfactin. The structures of viscosin and surfactin are shown in Figs. 1A and 1B. Both are lipopeptide biosurfactants which are synthesized non-ribosomally by multi-functional peptide synthetases. (Stachelhaus et al., *Biochemical Pharmacology* 52: 177-186 (1996)). Tests using pure viscosin and surfactin obtained from third-party sources confirmed the ability of these compounds to act as inhibitors of human c-Src kinase, a tyrosine kinase and to a lesser extent as inhibitors of the phospholipid dependent serine-threonine kinase, Protein Kinase C (PKC).

Surfactin and viscosin are best known for their ability to partition preferentially at interfaces; that is, both are surface-active compounds classed as biosurfactants. *Bacillus subtilis* and *Bacillus licheniformis* are well characterized producers of surfactin while *Pseudomonas fluorescens* is a known producer of viscosin. Both of these biosurfactants are lipopeptides known to have antibiotic properties which are probably related to their membrane acting characteristics. (review: Georgiou et al., *Biotechnology* 10: 60-65. (1992)). This same activity contributes to lysis of erythrocytes by surfactin. In a screen which was designed to detect compounds acting on hormonal signal transduction or cell-cycle regulation, bacterial culture supernatants were screened for their ability to inhibit maturation of starfish oocytes (Toraya et al., *Applied and Environmental Micro.* 5:1799-1804 (1995)). A positive result was obtained from a supernatant from a *Bacillus* species and following purification the active compound was identified as surfactin. The biochemical basis for the inhibition of oocyte maturation by surfactin has not yet been

- 5 -

determined and these authors make no suggestions or claims that surfactin may be acting as a kinase inhibitor. It should be noted, however, that it is possible that a kinase-inhibition activity may be responsible for the maturation inhibition process since this developmental process, like sporulation, is regulated by kinase-mediated signaling cascades.

The present invention provides recognition of a new class of compounds, lipopeptide biosurfactants, which may be used as inhibitors of eukaryotic protein kinases for investigational and therapeutic purposes. While the invention is exemplified through two specific lipopeptide biosurfactants, namely the cyclic depsipeptides surfactin and viscosin, other lipopeptides might also be employed including serrawettin.

The invention will now be further described and illustrated by way of the following, non-limiting examples.

EXAMPLE 1

For isolation of the active inhibitor from the supernatant of *Bacillus* 60A, a 3 litre culture of *Bacillus* 60A was grown in TSB (tryptic soy broth, Difco) for 24 hours at 30°C from a 1% v/v inoculum from a freshly grown seed culture. Cells were harvested by centrifugation at 7880 x g and both the supernatant and cell pellet were extracted separately with 3 volumes of ethyl acetate and 3 volumes of 20% methanol in ethyl acetate, respectively. The crude ethyl acetate extracts were fractionated on a low mesh silica gel column in an increasing methanol gradient in chloroform. Inhibitory activity in the fractions was monitored by thin layer chromatography of aliquots on silica gel. Following development in chloroform:methanol (95:5 v/v) plates were transferred to square culture dishes and overlaid with ISP (Difco) soft agar (0.6 %) containing an inoculum of 10⁷-10⁸ cfu from a fresh culture of *Streptomyces* 85E. After incubation at 30°C for 24 to 30 hours fractions with activity could be identified by inhibition of sporulation in a zone over one or more spots. Using this method an active fraction eluting at 12% methanol in chloroform was identified and further purified by column chromatography on Sephadex LH-20 in chloroform:methanol (1:1 v/v). Once again the active fraction was identified and chromatographed once more on Sephadex LH-20, this time in chloroform:methanol 8:2 v/v. 44.2 mg of a pure, active compound designated

- 6 -

TDI-4 were recovered. TDI-4 is a white powder, soluble in chloroform, which develops as a pink spot when sprayed with vanillin-H₂SO₄ following TLC in chloroform: methanol: acetone, 4:3:3 v/v. At this stage it was subjected to low resolution FAB-mass spectroscopy which gave M⁺ at m/z 1036. High resolution FAB-MS suggested the formula C₅₃H₉₃N₇O₁₃. Given the source of this compound, its molecular weight and probable formula an identification as the peptide surfactin seemed likely. Amino acid analysis yielded a composition of leucine x 4, aspartic acid x 1, glutamic acid x 1 and valine x 1, a composition consistent with surfactin. TDI-4 was then directly compared to surfactin from *Bacillus subtilis* (obtained from Sigma, molecular weight 1036) by co-TLC and by NMR spectroscopy and was found to match.

EXAMPLE 2

For isolation of the active inhibitor from *Pseudomonas* 11C, a 10 litre culture of *Pseudomonas* 11C was grown in tryptic soy broth for 24 hours at 30°C from a 1% v/v inoculum from a freshly grown seed culture. Cells were harvested by centrifugation at 7880 x g and both the supernatant and cell pellet were extracted separately with ethyl acetate and 20% methanol in ethyl acetate, respectively. As before, the crude ethyl extracts were fractionated initially on a silica gel column in chloroform, followed by further chromatography of active fractions (identified by TLC and an overlay bioassay as described previously) on Sephadex LH-20 in methanol. 4.2 mg of a pure active compound designated TDI-5 were recovered. TDI-5 is a yellowish white compound which also develops as a pink spot on a TLC plate when developed in chloroform:methanol:acetone (6:2:2) and sprayed with vanillin-H₂SO₄. The LRFAB-MS analysis suggested a molecular weight of 1126. The molecular formula for m/z 1126 was determined to be C₅₄H₉₆N₉O₁₆ by HRFAB-MS analysis. Based on this information and the source of the compound, an identification as the peptide viscosin seemed likely and amino acid analysis yielded a composition of leucine x 3., valine x 1, serine x 2, isoleucine x 1 and glutamic acid x 1. The presence of a ninth amino acid as D-allo-threonine was demonstrated by the mass fragmentation data calculated from LRFAB-MS. TDI-5 was then compared to an authentic sample of viscosin (a gift from Raymond Anderson) by co-TLC and found to match.

- 7 -

EXAMPLE 3

Testing the purified surfactin and viscosin was carried out to determine a) their ability to inhibit sporulation of *Streptomyces* species and b) their ability to inhibit the activity of eukaryotic protein kinases.

a) Inhibition of sporulation:

Samples of surfactin prepared as described from *Bacillus* 60A as well as surfactin from *Bacillus subtilis* with the same molecular weight (1036) obtained from Sigma were tested for their ability to inhibit sporulation of *Streptomyces* 85E.

Amount of surfactin applied to the disc	Diameter of zone of inhibition of sporulation
1 ug, Sigma surfactin	12 mm
5 ug, Sigma surfactin	15 mm
10 ug, Sigma surfactin	20 mm
1 ug surfactin from <i>Bacillus</i> 60A	13 mm
5 ug surfactin from <i>Bacillus</i> 60A	17 mm
10 ug surfactin from <i>Bacillus</i> 60A	19 mm

Surfactin in these concentrations does not inhibit growth of the vegetative mycelia but inhibits development of aerial mycelia and spore formation. The effect is very persistent, lasting for at least 3 to 4 days after unaffected portions of the culture have sporulated. Eventually the inhibition is overcome and the culture sporulates. It has also been noted that surfactin in these concentrations does not inhibit growth of other bacterial species such as *E. coli* or *S. aureus*. However, surfactin has been reported to have anti-mycobacterial activity (Stachelhaus *et al.*, 1996) and this has been confirmed with tests of these samples on *Mycobacterium phlei*. (25 ug Sigma surfactin applied to a disc produced a 12 mm zone of growth inhibition and 20 ug of surfactin from *Bacillus* 60A produced an 8 mm zone of growth inhibition.) The mechanism of this inhibition is not known; however, Mycobacteria have also been shown to possess certain elements of eukaryotic signal transduction pathways (Av-Gay and Davies, 1997) and it is possible that surfactin, acting as a kinase inhibitor, is interfering with signaling processes in this organism. It is further possible that the screen described which potentially detects inhibitors of eukaryotic

- 8 -

signaling processes may also have potential in the discovery of novel antimycobacterial agents. At least 8 cell-free supernatants which were found to inhibit sporulation of *Streptomyces* 85E also have antimycobacterial activity in tests of inhibition of *M. aurum*, *M. phlei* and *M. tuberculosis* but do not inhibit growth of *S. aureus* or *E. coli*. These active supernatants are from two species of *Streptomyces*, a *Bacillus* species and a *Pseudomonas* species isolated from soils. The active compounds from the *Streptomyces* sp. have not yet been purified and identified. The *Bacillus* species are related to 60A and are probably producing surfactin while the *Pseudomonas* is a viscosin producer.

Viscosin purified from *Pseudomonas* 11C was also shown to inhibit sporulation of *Streptomyces* 85E (15 ug produces a 30 mm zone of persistent sporulation inhibition with no inhibition of growth of vegetative mycelia) Viscosin has also been reported to have antimycobacterial activity (Gerard et al., 1997) and this was confirmed with inhibition of growth of *M. aurum*. (25 ug applied to a disc resulted in a 9 mm zone of growth inhibition.)

b) Inhibition of eukaryotic protein kinases:

I) Inhibition of the protein tyrosine kinase c-Src:

Human c-Src (p60^{c-src}) was obtained from Upstate Biotechnology. The activity of the enzyme was assayed in an ELISA based assay developed by Pierce. Following their protocol, 500 ng of a biotinylated peptide substrate in distilled water (Pierce biotinylated peptide substrate #2, sequence Biotin-EGPWLEEEEEAYGWMDF) was added to a NeutrAvidinTM coated well, incubated at 37°C for 30 minutes to allow binding to the well then washed with Tris-buffered saline (TBS, 25mM Tris, 0.15 M NaCl, pH 7.6). For each reaction, a reaction buffer mixture was prepared to contain 10 ul of src assay buffer (100 mM Tris-HCl, pH 7.2, 25mM MnCl₂, 2.0 mM EGTA, 0.25 mM Na₃VO₄, 125mM Mg Acetate) 20 ul of ATP/MgCl₂ buffer (5 mM ATP, 50 mM MgCl₂ in TBS) and 1 ul of protease inhibitor mix (PMSF and pepstatin, final concentrations in Rx: 0.5 mM and 1.5uM). The src enzyme is supplied at a concentration of 3 units per microlitre and dilutions of the kinase were prepared in 1% bovine serum albumin (BSA) in TBS typically to final concentrations of 0.2 or 0.4 units in 10 ul. 10 ul of a solution of a compound to be tested for inhibitory activity was added to the reaction buffer mixture and allowed to stand

- 9 -

10 -15 min. at room temperature, then 10 ul of the diluted kinase enzyme was added and the mixture was allowed to stand 10 min. at room temperature before addition to the substrate coated well. The plate was incubated at 30°C for varying periods of time then the contents were washed out with repeated rinsings with TBS. A mouse monoclonal anti-phosphotyrosine antibody (PY20) conjugated to horseradish peroxidase (Pierce) was diluted 1:2000 in 1% BSA diluent from 1 mg/ml and 75 ul of this dilution was added to each well, incubated 1 hour at 37°C then washed out with repeated rinsings with TBS.

100 ul of horseradish peroxidase substrate solution (3,3',5,5' tetramethylbenzidine and H₂O₂, Pierce TMB-ELISA) was added to each well and after 10 min. the reaction was stopped with the addition of 100 ul of 1 N H₂SO₄. Absorbance was read at 450 nm in a Molecular Devices V-max plate reader.

Examples of inhibition detected using this assay: (Values reported are the absorbance readings after subtraction of a blank well containing no enzyme.)

I. Human c-Src kinase incubated 30 min. at 30°C with 10 ug of surfactin (srf) or 5 ug of viscosin (vis):

<u>Control reactions</u>	<u>Reactions with surfactin</u>	<u>Reactions with viscosin</u>
0.05 unit c-Src 0.315	0.05 unit c-Src + 10 ug srf 0.050	
0.1 unit c-Src 0.391	0.1 unit c-Src + 10 ug srf 0.093	0.1 unit c-Src + 5 ug vis 0.186
0.2 unit c-Src 0.396	0.2 unit c-Src + 10 ug srf 0.191	0.2 unit c-Src + 5 ug vis 0.175
0.4 unit c-Src 0.517	0.4 unit c-Src + 10 ug srf 0.311	0.2 unit + 5 ug vis 0.251

II. Human c-Src kinase incubated 5 min. at 30°C with surfactin or viscosin:

Control

- 10 -

0.2 unit c-Src	0.2 unit c-Src + 10 ug srf	0.2 unit c-src + 5 ug vis
0.220	0.087	0.123
	0.2 unit c-Src + 5 ug srf	0.2 unit c-Src + 2.5 ug vis
5	0.185	0.210

10 ug of surfactin added to these reactions represents a concentration of 200 uM in the reaction and this concentration as well as 100 uM has inhibited the activity of the tyrosine kinase tested. Similarly viscosin at a concentration of 90 uM has inhibited the activity of this kinase. The preparations of the inhibitors from the soil bacterial isolates as well as the Sigma preparation of surfactin have been tested for residual protease activities by various methods including:

- a. incubation with the chromogenic substrate N-succinyl-ala-ala-pro-phe p-nitroanilide
- b. incubation with a mixture of proteins followed by SDS-PAGE analysis
- 15 c. zymogram analysis in a gel containing gelatin as the protease substrate

The crude culture supernatant from the Bacillus clearly contains protease activity but none was detectable in the purified preparations of surfactin. The Pseudomonas culture supernatant contains a lesser amount of protease activity and again none was detected in the purified preparation of viscosin. Both of these compounds are surfactants and the possibility that the apparent inhibition was due to interference with either the avidin-biotin interaction or the adherence of the avidin to the wells was tested by preincubating the substrate coated well with a reaction mixture containing surfactin or viscosin but no enzyme for 30 minutes. The well was then washed with repeated rinsings of TBS and a standard kinase reaction without the inhibitors was set up. This reaction showed no decrease in activity compared to a control reaction. Thus, it is concluded that surfactin and viscosin are inhibitors of this tyrosine kinase.

ii) Inhibition of the phospho-lipid dependent serine-threonine kinase Protein Kinase C (PKC)

Protein Kinase C purified from rat brain was obtained from Upstate Biotechnology. The activity of the enzyme was assayed in a colorimetric assay developed by Pierce. Reaction mixtures were prepared to contain 5 ul each of the 5X reaction buffer,

- 11 -

the dye labeled glycogen synthase synthetic peptide substrate reconstituted to 1.77 mM and the 5X activator solution of phosphatidyl-L-serine, all supplied by Pierce in a PKC assay kit. The activator solution was sonicated for 30 sec. on ice prior to use. 1 ul of the kinase (equivalent to 10 ng of enzyme with a specific activity of 12.1 units/mg) was added to 10
5 ul of a solution containing the compound to be tested for inhibitory activity and allowed to stand at room temperature for 10 minutes then transferred to the reaction tube containing the substrate and incubated 30 min. at 30°C. After incubation 20 ul of each sample was applied to the centre of the affinity membrane in the separation units supplied with the assay kit. These membranes specifically bind the phosphorylated dye labeled substrate
10 while non-phosphorylated peptide will pass through when spun at low speeds. After washing the membranes the phosphorylated peptide was eluted by spinning with the elution buffer and absorbance read at 570 nm in a Molecular Devices V-max plate reader.

Viscosin at a concentration of 200 uM in the reaction was not inhibitory. Surfactin at a concentration of 380 uM in the reaction was only minimally inhibitory as
15 shown in the following example:

Control reaction

10 ng PKC	10 ng PKC + 10 ug srf
0.228	0.181

20 Polymyxin B is a peptide with a molecular weight similar to viscosin and surfactin and is a recognized inhibitor of PKC. Inhibition of the kinase could be detected in this assay system as shown by the following example:

Control reaction

10 ng PKC	10 ng PKC + 10 ug polymyxin B
0.203	0.035

Thus it was concluded that surfactin and viscosin are more potent inhibitors of the tyrosine kinase tested than this serine-threonine kinase.

- 12 -

CLAIMS

1. A method for inhibiting eukaryotic protein kinase activity present in a sample or organism comprising the step of adding to the sample or organism an effective inhibitory amount of a lipopeptide biosurfactant that inhibits sporulation of *Streptomyces*.

5

2. The method of claim 1, wherein the lipopeptide biosurfactant is a cyclic depsipeptide.

10

3. The method of claim 2, wherein the lipopeptide biosurfactant is surfactin.

4. The method of claim 2, wherein the lipopeptide biosurfactant is viscosin.

15

1/1

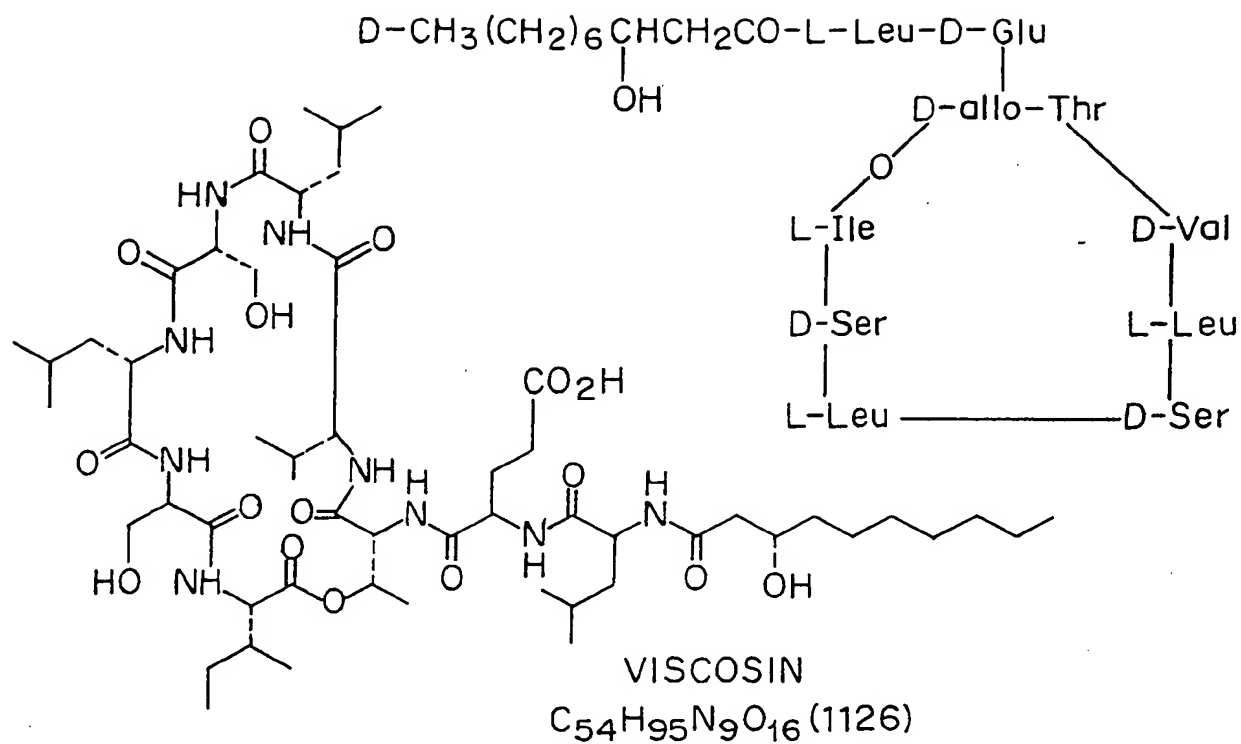


FIG. 1A

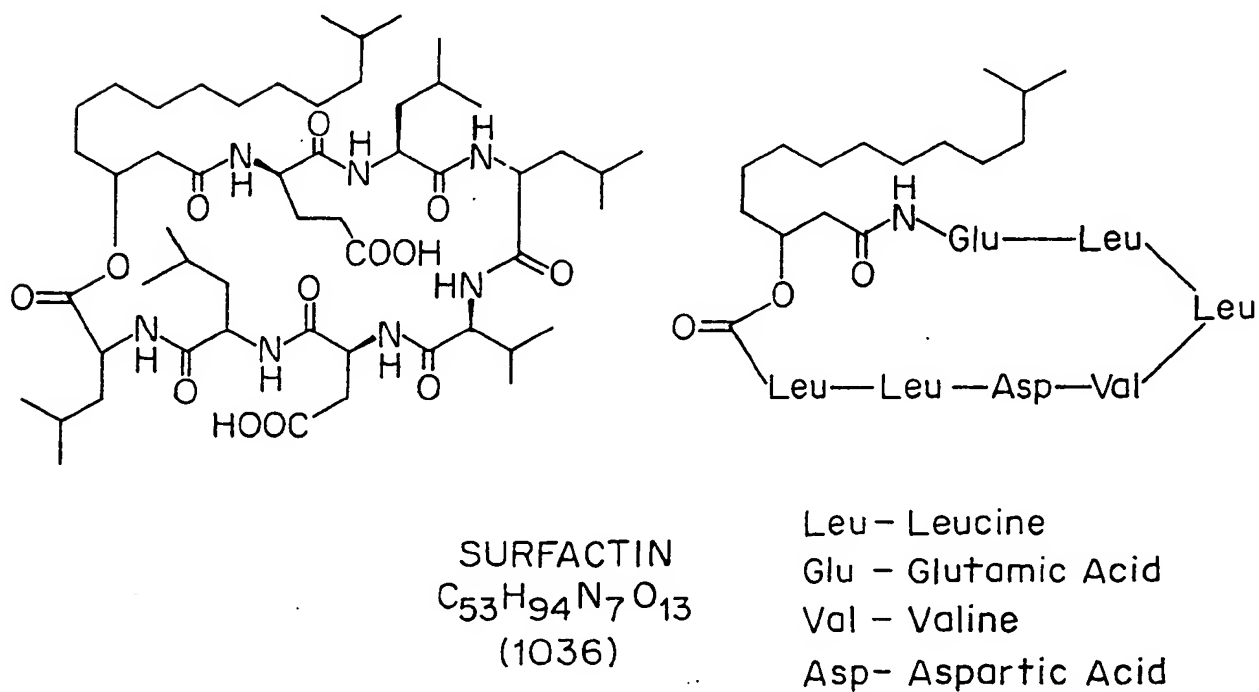


FIG. 1B

INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 98/00970

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12Q1/48 C12Q1/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	WO 98 17822 A (TERRAGEN DIVERSITY INCORPORATED) 30 April 1998 cited in the application see example 13 -----	1-4



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

2 February 1999

Date of mailing of the international search report

11/02/1999

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Van Bohemen, C

...ormation on patent family members

PCT/CA 98/00970

Form PCT/SA/210 (patent family annex) (July 1992)